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Stressed tadpoles mount more efficient glucocorticoid negative feedback in anthropogenic habitats due to phenotypic plasticity

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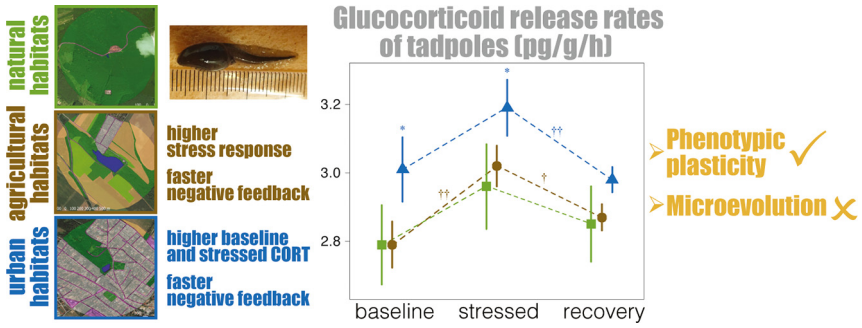
HIGHLIGHTS

- Endocrine flexibility likely helps organisms cope with anthropogenic habitat change.
- We tested if land use, water pollution, and pathogens affect glucocorticoid profiles.
- We sampled tadpoles in agricultural, urban, and natural ponds and in "common garden".
- Stress response and negative feedback were upregulated in anthropogenic habitats.
- Negative feedback is key to understanding how animals adapt to the anthroposphere.

GRAPHICAL ABSTRACT



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ABSTRACT

Coping with anthropogenic environmental change is among the greatest challenges faced by wildlife, and endocrine flexibility is a potentially crucial coping mechanism. Animals may adapt to anthropogenic environments by dampening their glucocorticoid stress response, but empirical tests of this hypothesis have provided mixed evidence. An alternative hypothesis is that a non-attenuated stress response and efficient negative feedback are favored in anthropogenic habitats. To test this idea, we non-invasively sampled corticosterone release rates of common toad (*Bufo bufo*) tadpoles in agricultural, urban, and natural habitats, and quantified their stress response and negative feedback by a standardized stress-and-recovery protocol. We repeated the same sampling with tadpoles raised from eggs from the same ponds in a common-garden experiment to infer if the differences observed between populations in different habitats were due to individual phenotypic plasticity rather than microevolution or transgenerational effects. We found that, compared to tadpoles in natural ponds, urban tadpoles had higher baseline and stressed corticosterone release rates, and tadpoles in agricultural ponds had similar corticosterone release rates but greater stress-induced change, indicating stronger stress responses in both types of anthropogenic habitats. As predicted, tadpoles in both agricultural and urban ponds showed more efficient negative feedback than did tadpoles in natural ponds. Water pollution levels, as indicated by the concentrations of carbamazepine and corticoid-disrupting compounds in pond water, contributed to elevating the stress response

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regardless of land use. Infection by neither *Batrachochytrium dendrobatidis* nor *Ranavirus* was detected in free-living tadpoles. No habitat-related glucocorticoid differences persisted in the common-garden experiment. These results suggest that toad tadpoles in anthropogenic habitats increased their glucocorticoid flexibility via phenotypic plasticity. The coupling of stronger stress response and stronger negative feedback in these habitats supports the importance of rapidly "turning on and off" the stress response as a mechanism for coping with anthropogenic environmental change.

1. Introduction

Anthropogenic environmental change exposes wildlife to many kinds of stressors: unpredictable or uncontrollable stimuli that threaten the organism's life or homeostasis. For example, both urban and agricultural land use loads the environment with various chemical pollutants, which are accompanied in urban areas by noise, light pollution, and the urban heat island effect (Bókony et al., 2018; Mann et al., 2009; Seress and Liker, 2015). Understanding the phenotypic and genetic adaptations by which organisms cope with these challenges is an important area of current research in ecology, evolutionary biology, and conservation (Bonier, 2012; Guindre-Parker, 2018; Liker, 2020).

The glucocorticoid (GC) response to stress in vertebrate animals may play a crucial role in organismal adaptation to anthropogenic environments (Bonier, 2012; Partecke et al., 2006). Glucocorticoids are secreted in response to stressful stimuli by the activation of the hypothalamus-pituitary-adrenal/interrenal (HPA/I) axis (Romero, 2004; Romero et al., 2009; Sapolsky et al., 2000) and mediate a wide variety of physiological and behavioral changes that help animals cope with stressors (Sapolsky et al., 2000). However, when a stressor persists and cannot be avoided, GCs can lead to pathological effects that directly impact fitness and can ultimately result in death (Romero and Wikelski, 2010; Wingfield and Sapolsky, 2003). In such situations, dampening of the stress response or "resistance to stress" may be adaptive (Wingfield and Sapolsky, 2003). Researchers have hypothesized that wildlife populations in anthropogenic environments can escape the negative effects of chronic stress by reducing their responsiveness to stressors (Atwell et al., 2012; Partecke et al., 2006). This hypothesis has generated great interest, yielding a large amount of mixed results across species, mostly in birds (Bonier, 2012; Sepp et al., 2018) and in reptiles (French et al., 2018). That is, urban populations in some species show higher stress response than non-urban conspecifics while other species show the opposite or no difference between habitats; the reason for this heterogeneity could not yet be identified by interspecific comparative studies and meta-analyses (Injaian et al., 2020; Murray et al., 2019).

Glucocorticoid negative feedback is a key component of the stress response that needs investigation to understand if and how the modulation of the stress response helps organisms cope with anthropogenic habitats (Narayan et al., 2019). The stress response involves suppressive actions on the HPA/I axis which decrease the production of GCs; thus, an efficient negative feedback returns GC levels to their baseline quickly after cessation of the stressor (Sapolsky et al., 2000). According to the reactive scope model of stress, the shorter the duration of the acute stress response, the lower the chances that repeated or prolonged stressors will provoke phenotypic damage; so an efficient negative feedback may allow for a strong stress response without leading to pathology (Romero et al., 2009). Negative feedback efficiency varies between individuals and is modulated by environmental conditions (Lattin and Kelly, 2020; Vitousek et al., 2019). Individuals with more efficient negative feedback are less likely to suffer from chronically elevated GC levels (Taff et al., 2018) and are better able to cope with stressors such as starvation (Romero and Wikelski, 2010), predation risk (Zimmer et al., 2019), or disturbance by noise (Soldatini et al., 2015). Therefore, we hypothesize that a non-attenuated stress response coupled with efficient negative feedback may help wildlife adapt to anthropogenic habitats (Narayan et al., 2019; Vitousek et al., 2019; Wingfield, 2013), predicting faster recovery from acute stress in individuals in these habitats. Such a strategy might be an alternative to a suppressed stress response; thus, the heterogeneity in empirical findings might be due to different species relying on different coping strategies. So far, only a single study reported empirical data for evaluating this idea, finding that urban curve-billed thrashers (*Toxostoma curvirostre*) were more responsive to stress (adrenocorticotropin hormone, ACTH injection) than conspecifics in a desert population, but negative feedback as measured by a dexamethasone suppression test did not differ between the two habitats (Fokidis and Deviche, 2011).

Another potential reason for heterogeneity in stress responsiveness in relation to anthropogenic environments is that these environments are highly heterogeneous in the ways that they are impacted by human activities. For example, infection by parasites and pathogens can influence GC levels (Gabor et al., 2013; Raouf et al., 2006; Warne et al., 2011) and may vary among habitats with anthropogenic influence (Davis et al., 2020; St-Amour et al., 2008) or independently of it, for example with elevation (Gabor et al., 2015). Chemical contaminants may further complicate the picture. Due to pollution by wastewater, pharmaceutical discharge, and manure in agricultural areas, natural water bodies often contain GCs and other hormonally active compounds (Gabor et al., 2018; Lange et al., 2002; Macikova et al., 2014). Such water-borne pollutants are taken up by wild animals, especially by those living in aquatic habitats, and may disrupt their endocrine system (Lange et al., 2002; Macikova et al., 2014; Pottinger, 2017). Although anthropogenic habitats are generally more polluted, endocrine disrupting chemicals occur in natural habitats as well (Bókony et al., 2018). Therefore, while chemical contaminants in general may act as physiological stressors, corticoid-disrupting compounds may have specific effects on the stress response and its negative feedback, which might confound the responses of the HPA/I axis to other anthropogenic stressors. For example, progesterone, an endogenous hormone that is also used as medication, occurs in wastewaters as well as natural water bodies and can affect various components of the GC system, including expression of GC receptors and enzymatic regulation of GC biosynthesis (Macikova et al., 2014). Together it is clear that exploring possible stressors across habitats will provide a greater understanding of the variable responses of organisms to anthropogenic habitats.

Phenotypic differences, such as those observed in stress physiology, may arise between individuals occupying different habitats by various mechanisms. Adaptive changes may come about by two, non-mutually exclusive processes: phenotypic plasticity within individuals, and changes in population composition due to genetic differentiation by natural selection or other trans-generational effects (Donelan et al., 2020) such as epigenetic variation (Taff et al., 2019). For simplicity, we refer to the latter group of processes as "persistent effects" (as opposed to individual plasticity). The role of plastic and persistent mechanisms in phenotypic divergence between populations can be tested with common garden (or transplant) experiments, whereby individuals from different populations are reared in a common environment (Carroll and Fox, 2008; De Villemereuil et al., 2016; DeWitt and Scheiner, 2004; Guindre-Parker, 2018). Such experiments have shown that both individual plasticity and persistent population divergence can contribute to differences in baseline GC levels and stress response between urban and non-urban habitats (Atwell et al., 2012; Ouyang et al., 2019; Partecke et al., 2006). No such study to our knowledge

has additionally assessed the role of plastic versus persistent mechanisms in differences of GC negative feedback efficiency between populations living in habitats with different levels of anthropogenic influence.

In this study, we had three main objectives. First, we examined whether the GC stress response and its negative feedback differed between anthropogenic and natural habitats. Second, we investigated whether these differences were associated with pathogenic infections and chemical contaminants. Third, we tested the role of persistent population divergence in the different GC profiles observed between habitats. To address these questions, we studied tadpoles of the common toad (*Bufo bufo*), an anuran species that occurs throughout Europe in various habitats. Amphibians have rarely been considered in research of the differences in GCs in relation to anthropogenic land use (Davis et al., 2020; Gabor et al., 2018; Goff et al., 2020; Hopkins and DuRant, 2011; Janin et al., 2011; Orton et al., 2014), despite the fact that human-induced environmental change is among the most likely causes of their global population declines (Mann et al., 2009). Here we tested 1) whether tadpoles living in natural, agricultural, and urban habitats differ in their release rates of corticosterone (the main GC of amphibians) in response to a standardized stressor, and after a standard time allowed for recovery (i.e., negative feedback); 2) whether stress response and negative feedback are altered in habitats with high levels of water pollution, in general, and corticoid disruptors specifically, or high levels of infection by two important groups of anuran pathogens, *Ranavirus* and *Batrachochytrium dendrobatidis* (*Bd*); and 3) whether the differences observed in the field persist when individuals from different habitats are raised in a common garden experiment, which would indicate persistent population divergence rather than phenotypic plasticity as the main mechanism of GC responses to anthropogenic environments.

2. Methods

All procedures in this study were in accordance with animal ethics guidelines and approved by the Ethics Committee of the Plant Protection Institute, Centre for Agricultural Research. Permit for the study was issued by the Environment Protection and Nature Conservation Department of the Pest County Bureau of the Hungarian Government (PE-06/KTF/8060-3/2018, PE-06/KTF/8060-1/2018, PE/EA/295-7/2018).

2.1. Study sites and sampling in the field

We selected three ponds per each of three different habitat types in North-central Hungary (Table 1). Three ponds were surrounded by woodlands, with no arable fields or residential areas in a 500-m wide buffer zone around the pond (Table 1, Fig. S1a); these are considered natural habitats for common toads. Three ponds were in agricultural landscapes with 22–48% arable fields and <7% residential areas, whereas another three ponds were in urban landscapes with 29–72% residential areas and <3% arable fields (Table 1, Fig. S1a); we will refer to these as agricultural and urban habitats, respectively. The percentage of each land-use type in the 500-m wide buffer zones around the ponds was measured using QGIS as in an earlier study (Bókony et al., 2018). We chose these ponds based on earlier data that indicated common toads breed there in sufficiently large numbers and with similar seasonal timing (Bókony et al., 2018).

We sampled common toad tadpoles at each of the nine ponds between 9 and 20 May 2019, in the early phase of larval development, before marked differentiation of toes. We estimated that the majority (90%) of sampled tadpoles were between developmental stages 28–31 (Gosner, 1960), and had spent at least 5 weeks in their habitat since spawning. Each pond was sampled on a different day between 0930 and 1430 h; date and time of sampling were unbiased by habitat type. We used a non-invasive method of water-borne hormone sampling to take a baseline, stressed, and recovery sample from each tadpole. This method provides an integrated measure of corticosterone which is repeatable within individuals, correlates with plasma levels, and responds to ACTH challenge (Forsburg et al., 2019; Gabor et al., 2016, 2013; Narayan et al., 2019); the post-stress rate of recovery of GC levels provides a measure of natural negative feedback (Lattin and Kelly, 2020). Upon sampling, we quickly collected 18 tadpoles from the pond with a dip net, and placed each tadpole in a clean plastic insert (a perforated cup, to facilitate removal of tadpoles from beakers) in a 250 ml glass beaker containing 100 ml spring water. The animals were left undisturbed for 1 h to measure "baseline" corticosterone release rates, after which we moved them (with the perforated insert) into another beaker of 100 ml spring water. Over the next hour, the tadpoles were agitated by gently shaking their beakers for 1 min every 3 min (Forsburg et al., 2019) to measure "stressed" corticosterone release rates, then moved into a third beaker with 100 ml spring water and left undisturbed again for an hour to measure "recovery" corticosterone release rates. The three water-borne hormone samples of each animal, and a 100-ml sample of pond water for measuring the background levels of corticosterone (Gabor et al., 2018), were filtered in the field using coffee filters (equivalent to grade 4 filter paper) immediately after collection, stored on ice in the dark for 3–5 h while being transported into the lab, and placed in −20 °C before we measured corticosterone shortly thereafter. Throughout hormone sampling we wore non-powdered nitrile gloves and we cleaned the beakers and inserts with 96% ethanol and rinsed them with reverse-osmosis filtered water before each use. After sampling, we transported the tadpoles to the lab in individual capped containers, measured their body mass (±0.1 mg), euthanized them by cooling-then-freezing (Shine et al., 2019), and preserved each animal in 1 ml 70% ethanol.

During hormone sampling, we measured water temperature, pH, conductivity, total dissolved solids, and salinity in the pond at the location where we collected the tadpoles, using a portable electrochemistry meter (Consort C 6020 T; Consort Ltd., Turnhout, Belgium). Using test strips (WaterWorks 480009), we tested for nitrite and nitrate in the pond water (detection limits: 0.495 mg/l for nitrite, 2.2 mg/l for nitrate). Before leaving the pond, we took a 1-l sample of pond water in an amber PET flask for measuring the concentrations of polluting chemicals; this sample was kept in the dark and on ice during transportation, and then stored at −80 °C until analysis.

2.2. Common garden experiment

On 12 March 2019, we set up 54 mesocosms by placing 45-l plastic tubs (56 × 39 × 28 cm) in an open outdoor area and filling them with 40 l tap water. Two days later we added 0.5 l pond water and 40 g dried beech (*Fagus sylvatica*) leaves to each tub to set up a self-sustaining ecosystem that provided nutrients and refuge for tadpoles. We repeated the pond-water inoculation two weeks later to ensure a sufficiently large population of phytoplankton and zooplankton. To prevent colonization by predators, we covered the tubs with mosquito net lids, and we removed all mosquito larvae from the pond water before inoculation.

Between 3 and 5 April, we collected common toad eggs from four freshly spawned egg strings (ca. 30 eggs per egg string) from each of the nine ponds, and transported them to our laboratory, where they were raised until developmental stage 25 (Gosner, 1960). Each family was kept in a separate plastic box (19 × 30 × 15 cm) in 2 l reconstituted soft water (48 mg $NaHCO_3$, 30 mg $CaSO_4 \times 2\ H_2O$, 61 mg $MgSO_4 \times 7\ H_2O$, 2 mg KCl added to 1 l reverse-osmosis filtered, UV-sterilized tap water). Lab temperature was 19 °C and we maintained a light-dark cycle that mimicked the natural photoperiod. On 13 April, we haphazardly selected 9 tadpoles from each family, mixed together the 36 tadpoles that originated from the same pond, and randomly distributed them among 6 mesocosms for each pond, resulting in 6 tadpoles per mesocosm and a total number of 324 tadpoles in 54 mesocosms. Pond of origin was allocated to the mesocosms in a randomized block design.

Table 1
The studied ponds' geographical coordinates, habitat type (N: natural, A: agricultural, U: urban), proportion of each major type of land-use cover in a 500-m wide belt around the pond, and concentration of pollutants detected in the pond water at the time of tadpole sampling (CARB: carbamazepine, TEST: testosterone, PROG: progesterone, HCOR: hydrocortisone or cortisol, FLUO: fluocinolone acetonide; values that are below but close to the limit of quantification and well above the limit of detection are marked with asterisk).

Pond	Geographical coordinates (latitude, longitude)	Percentage of land-use cover (%)						Habitat type	Concentration (ng/l) of pollutants				
		Forest	Pasture	Arable field	Residential area	Road	Industrial area		CARB	TEST	PROG	HCOR	FLUO
Apátkút	47.77444, 18.98624	88.8	8	0	0.7	1.8	0.8	N	0.860	0.219	<0.2	<1	<2
Bajdázó	47.903382, 18.978482	97.0	2.2	0	0	2.4	0	N	<0.2	<0.2	<0.2	0.646	1.573*
Szarvas-tó	47.881829, 18.942020	97.3	0	0	0	1.5	1.2	N	<0.2	<0.2	<0.2	<1	<2
Határrét	47.646376, 18.909233	28.4	13.7	48.4	7	2.6	0	A	175.667	<0.2	<0.2	<1	<2
Juliannamajor	47.551195, 18.926707	63.5	9.6	21.6	2	1.5	1.8	A	0.175*	0.355	0.266	<1	1.657*
Perőcsény	47.986458, 18.841379	49.8	14.1	34.6	0	1.4	0	A	2.090	<0.2	<0.2	<1	<2
Nagykovácsi	47.576402, 18.868515	47.6	15.6	2.5	28.7	3.9	1.8	U	0.541	0.250	2.343	<1	<2
Pesthidegkút	47.569349, 18.954981	15.6	0	1.3	72.4	7.7	3.1	U	0.483	<0.2	<0.2	<1	<2
Pilisszentiván	47.606498, 18.909010	28.2	0	0	45.5	7.6	17.3	U	13.633	<0.2	0.323	<1	<2

Between 6 and 8 May, we took water-borne hormone samples from the tadpoles in the mesocosms using the same protocol as we applied in the field. At this time, the captive-reared tadpoles were in similar developmental stages as the free-living tadpoles were at hormone sampling. We sampled 3 tadpoles from each tub (18 tadpoles in total per pond of origin), totaling 162 tadpoles; 54 per day. Sampling took place between 0900 and 1330 h each day. The order of sampling of the tubs followed a randomized block design such that two tubs per pond were sampled each day, and both date and time of day were balanced among the three habitat types. After sampling, we measured the body mass (± 0.1 mg) of each tadpole and released them back to their tubs; after completion of the experiment, tadpoles were transported back to their ponds of origin. The water-borne hormone samples were filtered after removing the tadpoles and stored at −20 °C until measuring corticosterone.

2.3. Measuring corticosterone

We extracted corticosterone from the water samples following an established protocol (Gabor et al., 2016). Briefly, we extracted hormones from the water samples using C18 solid phase extraction (SPE) columns (SepPak Vac3 cc/500 mg; Waters, Inc., Milford, MA, USA) primed with 100% HPLC-grade methanol (4 ml) and distilled water (4 ml). We then froze the columns and transported them from Hungary to Texas. Once defrosted, we eluted columns with 4 ml methanol into borosilicate vials, followed by evaporation with nitrogen gas. After drying, we re-suspended the residue in a total volume of 500 μl consisting of 5% ethanol (95% lab grade) and 95% enzyme-immunoassay (EIA) buffer (Cayman Chemicals Inc., Ann Arbor, MI, USA).

We measured corticosterone concentration in duplicates for all samples using Corticosterone EIA kits (№ 501320, Cayman Chemical Company, Inc.; assay has a range of 8.2–5000 pg/ml and a sensitivity (80% B/B0) of approximately 30 pg/ml). Sample absorbance was read on a spectrophotometer plate reader at 405 nm (BioTek 800XS). Inter-plate variation was 14.3% for the field data (13 plates; range: 0.07–17.7%) and 9.28% for mesocosms (9 plates; range: 0.31–8.08%).

Both in the field and in the mesocosms, we sampled 162 tadpoles (18 per pond) to ensure adequate sample size allowing for accidental loss of samples due to spillage, problems during extracting through SPE or at the plating stage. Because of the high cost of SPE columns and EIA kits, we aimed to analyze 16 tadpoles per pond in the field and 12 tadpoles per pond in the mesocosms (we expected lower variance in the common garden experiment than in the field). However, our final sample sizes were somewhat lower (Table 2) due to sample loss during sample processing and measurement.

2.4. Detecting pathogen infections

All free-living tadpoles included in the analyses of corticosterone release rates were tested for *Bd*, and 30% were tested for *Ranavirus* (for sample sizes, see Table 2). Mouthparts and tail-clips of tadpoles were excised using sterile scalpels and preserved in 70% ethanol. Both tissue sections of the same animal were extracted together in the MNCN-CSIC lab (Madrid, Spain) by using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol. Then, two independent quantitative PCRs, one for *Bd* and one for *Ranavirus* were performed following Boyle et al. (2004) and Leung et al. (2017), respectively, on a MyGo Pro qPCR machine. Negative controls and standards with known concentrations of *Bd* and *Ranavirus* were used in each plate. A sample was considered positive when its infection load was equal to or higher than 0.1 genomic equivalents of zoospores for *Bd* or 3 virions for *Ranavirus* (the smaller standards we used), and the amplification curve presented a robust sigmoidal shape.

2.5. Measuring water pollutants

In the 1-l pond water samples we measured the concentration of 23 endocrine disrupting compounds that can affect glucocorticoid signaling pathways (see Table S1 for full list of compounds), including steroid hormones and pharmaceutical drugs that get into natural water bodies via wastewater and agricultural runoff (Lange et al., 2002; Macikova et al., 2014). Additionally, we measured the concentration of carbamazepine which is considered a general marker for wastewater pollution (Nakada et al., 2008; Tran et al., 2014). For all compounds, we applied a newly developed UPLC-UniSpray™ MS/MS analysis as follows.

Samples were collected into brown polyethylene terephthalate (PET) bottles at field sites then transferred to the laboratory where they were frozen and stored at −80 °C until preparation. Following thawing 40 ml of water was filtered through 0.22 μm PTFE syringe filters into 50 ml centrifuge tubes. Afterwards 5 ml of methanol including the internal standard (carbamazepine-d10; final concentration: 5 ng/L) with/without spiking standards in two levels (final concentrations: 5 and 25 ng/l) was added to reach the final volume of 45 ml. After vortexing, solid phase extraction was applied with SampliQ (C_{18}; 3 ml; 500 mg) cartridges (Agilent Technologies; Santa Clara, California, USA). Cartridges were conditioned first with 6 ml methanol, then with 3 ml of 10 v/v% methanol in water. After conditioning samples were loaded; when necessary slight vacuum was applied. Washing of cartridges was done with 3 ml of 10 v/v% methanol in water, while elution was done with 3 ml of methanol. Eluates were evaporated to dryness at 50 °C in a vacuum centrifuge. Samples were dissolved in two steps, first in 0.2 ml methanol followed by adding 0.2 ml water, and then they were

Table 2
Sample sizes: number of tadpoles with usable corticosterone data in the field/in the common garden experiment; and the number of free-living tadpoles tested for *Batrachochytrium dendrobatidis* (Bd)/*Ranavirus* (Rv).

Pond	Baseline	Stressed	Recovery	Stress-induced change	Negative feedback	Bd/Rv
	Corticosterone release rate					
Apátkút	16/13	16/13	16/12	16/13	16/12	16/8
Bajdázó	17/13	17/13	17/13	17/13	17/13	17/4
Szarvas-tó	16/14	15/13	16/12	15/13	15/12	16/5
Határrét	16/14	16/13	16/14	16/13	16/13	15/6
Juliannamajor	16/12	16/12	16/12	16/12	16/12	16/5
Perőcsény	16/13	14/14	16/13	13/13	13/13	16/4
Nagykovácsi	16/14	17/13	15/13	16/13	15/12	17/4
Pesthidegkút	16/14	16/15	16/13	16/13	16/13	15/5
Pilisszentiván	15/13	15/12	15/12	15/12	15/12	16/3
Total	144/120	142/118	143/114	140/115	139/112	144/44

vortexed and filtered through 0.22 μm PTFE syringe filters. Final sample concentration ratio was 100×.

Samples were injected into an Acquity I-class UPLC system connected to a Xevo TQ-XS Mass spectrometer (Waters; Milford, MA, USA) equipped with a BEH C_{18} column (100 × 2.1 mm; 1.7 μm; Waters). Injection volume was 5 μl and the autosampler temperature was set to 8 °C. Eluent "A" was H_2O, while eluent "B" was methanol containing 0.1 v/v% formic acid. The column was kept at 40 °C. Gradient elution at 0.3 ml/min was set as follows: 0 min 50% B, 0–8 min up to 64% B, 8–11 min up to 84% B, 11–12 min to 100% B, 12–15 min hold 100% B, 15–15.5 min return to 50% B, and finally 15.5–18 min equilibration with 50% B.

For 17-alpha-ethinylestradiol and 17-beta-estradiol, a different gradient setup with a shorter BEH C_{18} column (50 × 2.1 mm; 1.7 μm; Waters) was used: eluent "A" was H_2O with 0.05 v/v% ammonia and eluent "B" was methanol. Injection volume was 10 μl, and gradient elution at 0.5 ml/min was as follows: 0 min 30% B, 0–8 min up to 50% B, 8–8.25 min up to 95% B, 8.25–9 hold 95% B, 9–10 min equilibration at 30% B. All solvents and chromatographic reagents were UPLC-MS grade and purchased from VWR International (Radnor, Pennsylvania, USA), while water (18.2 MΩ·cm) was obtained with a Milli-Q system (Merck-Millipore, Burlington, MA, USA).

Compounds of interest were infused into the mass spectrometer and optimized for MRM experiments with a classic ESI source, while for measurements a UniSpray™ ion source was applied. Optimized MRM transitions are summarized in Table S2. Argon (5.0 purity) was used as collision gas with 0.15 ml/min in the collision cell and nitrogen was used as source gas. UniSpray™ source settings were as follows for both positive and negative modes: capillary voltage 2.5 kV; desolvation temperature 550 °C; desolvation gas flow 1000 L/h; cone gas flow 300 l/h; nebulizer pressure 6.5 bar. Data processing was carried out with MassLynx version 4.2 in TargetLynx software (Waters). For quantification, external calibration was used in the linear dynamic range of 0.05 ng/ml–10 ng/mL (0.5–100 ng/l) where R^2 was at least 0.998 (linear with 1/x weighting) for all components. Recovery tests were conducted at 5 and 25 ng/l levels for both chromatographic methods, as summarized in Table S2. Reference material purchased, purity and solvents used for stock solutions are listed in Table S3.

2.6. Statistical analyses

We quantified corticosterone release rates as the amount of waterborne corticosterone measured over 1 h, divided by tadpole body mass (pg/g/h), and we used its 10-base logarithm in our statistical analyses. We did not correct for developmental stage because body mass is correlated with developmental stage in young tadpoles, and corticosterone levels do not differ across Gosner stages 25–40 (Glennemeier and Denver, 2002). We used the R computing environment (v. 3.6.3) for all statistical analyses (R Core Team, 2020). First, we used a linear mixed-effects model (LMM; 'lme' function in package 'nlme') to test if tadpoles in the field and in the mesocosms showed stress response (i.e. significant difference between baseline and stressed corticosterone release rates) and negative feedback (i.e. significant difference between stressed and recovery corticosterone release rates). We used the three consecutive samples of each individual as repeated measures, and we tested the fixed effects of venue (field or mesocosms), sample (baseline, stressed, and recovery corticosterone release rate), and their interaction. Individual identity was included as a random factor. We calculated a type-2 analysis-of-deviance table to test the main effects of venue and sample, and their interaction, using the 'Anova' function in package 'car'. We extracted the model's estimated marginal means and compared pairwise the three categories of sample separately for the two venues, and corrected the significance level with the false discovery rate (FDR) method, using the 'emmeans' package.

Second, we tested if baseline, stressed, and recovery corticosterone release rates, and the magnitude of stress response and negative feedback differed among the three habitat types (natural, agricultural, and urban) separately in the two venues. We used two variables to quantify the magnitude of stress response: the absolute stressed levels of corticosterone release rates, and the relative change of corticosterone release rate in response to stress (stress-induced change: 100 × (stressed − baseline) / baseline). Similarly, we quantified negative feedback as the relative change from stressed to recovery levels as: 100 × (stressed − recovery) / stressed (Lattin and Kelly, 2020). Because the values of stress-induced change were strongly right-skewed whereas the values of negative feedback were strongly left-skewed, we applied power transformations (power ¼ for the former and power 4 for the latter) to ensure that the residuals conform to the assumptions of normality and homoscedasticity. Because both variables had negative values, for each variable the |minimum value − 1| was added to all values before power transformation.

For each of the five dependent variables (baseline, stressed, and recovery corticosterone release rates, stress-induced change, and negative feedback), we used the 'geeglm' function in the 'geepack' package to build two generalized estimation equations (GEE) models: one for the field data and one for the mesocosms. GEE is a population-averaging method that can handle the correlation structure of our data (i.e. tadpoles from the same pond are not independent, but the pond effect is nested within the habitat effect) appropriately and without penalizing power (Zuur et al., 2009). We analyzed the venues separately because the correlation structure as well as the relevant fixed effects differed between venues. In the field, pond identity was a significant random effect (Table S4), and the relevant fixed effects included the actual levels of pollution and date as a numeric covariate. In the mesocosms, tub identity was a significant random effect whereas pond of origin was not (Table S4), and date had only 3 different values so it could not be used as a numeric covariate; we did not consider the pollution levels (measured in the ponds one month after taking the eggs into the common

garden) as predictor for the tadpoles in the mesocosms. Therefore, in the GEE models of the field data, we tested the following fixed effects: habitat type, date (number of days since 1st May), time of day (number of hours since 0800 h), carbamazepine concentration, and total concentration of corticoid-disrupting chemicals; the latter two variables were transformed as $\log_{10}(x + 0.1)$ to ensure normal distribution and homoscedasticity of model residuals. There was no multi-collinearity between the predictors included in the models (variance inflation factor: VIF < 1.7); we did not include water-temperature and water-quality variables because of their multi-collinearity with the other predictors (VIF > 2.2). The random factor in these models was pond identity. In the GEE models of the mesocosms data, we tested the fixed effects of habitat type, date (as a 3-category factor), and time of day; the random factor was tub identity. All numeric predictors were mean-centered.

3. Results

Ponds in anthropogenic habitats tended to have higher water temperature, pH, conductivity, total dissolved solids, and salinity values compared to natural ponds (Figs. S1b, S2). We detected carbamazepine in all anthropogenic ponds and in one natural pond (Table 1). Out of the studied 23 corticoid disruptors, we detected 4 in at least one pond (Table 1); the number of chemicals detected per pond was unrelated to habitat type (Fisher's exact test: $P > 0.999$). Corticosterone concentrations in pond water were higher in agricultural than in urban ponds, but did not differ systematically between natural and anthropogenic ponds (Fig. S3). The concentrations of nitrite or nitrate were below detection limit in all ponds. None of the free-living tadpoles tested positively for either *Bd* or *Ranavirus*.

Corticosterone release rates were significantly affected by venue (LMM: $\chi^2 = 1168.9$, df = 1, $P < 0.001$), sample ($\chi^2 = 197.6$, df = 2, $P < 0.001$), and their interaction ($\chi^2 = 156.4$, df = 2, $P < 0.001$). Tadpoles mounted a significant stress response (stressed levels were significantly higher than baseline levels) and then showed significant negative feedback (recovery levels were significantly lower than stressed levels) both in the field and in mesocosms (Table 3, Fig. 1). The recovery levels were significantly higher than the baseline levels in the mesocosms but did not differ significantly from baseline in the field (Table 3, Fig. 1).

In the free-living tadpoles, we found several differences between natural and anthropogenic habitats (Table 4, Fig. 1). Tadpoles in urban ponds had higher baseline and stressed corticosterone release rates than tadpoles in natural ponds; their stress-induced change did not differ significantly (Table 4, Figs. 1, S4). In contrast, tadpoles in agricultural ponds had similar corticosterone release rates but greater stress-induced change compared to tadpoles in natural ponds (Table 4, Figs. 1, S4). Tadpoles in both urban and agricultural ponds exhibited stronger negative feedback, their corticosterone release rates returning to similar recovery levels, compared to tadpoles in natural ponds (Table 4, Figs. 1, S4). Furthermore, tadpoles in ponds with higher concentrations of corticoid disruptors had higher stressed and recovery corticosterone release rates and greater stress-induced change (Table 4, Figs. 2–3), and the rate of stress-induced change increased with carbamazepine concentration (Table 4, Fig. 3).

Table 3
Pairwise comparisons (*c: linear contrasts, estimated from linear mixed model*) of corticosterone release rates ($\log_{10}$ pg/g/h) between samples in each venue. *P*-values were corrected with the FDR method; df = 509.

Venue	Contrast	c ± SE	t	*P*
Field	Stressed–baseline	0.091 ± 0.029	3.13	0.003
	Stressed–recovery	0.104 ± 0.029	3.60	0.001
	Recovery–baseline	−0.014 ± 0.029	−0.47	0.636
Mesocosms	Stressed–baseline	0.54 ± 0.032	16.96	<0.001
	Stressed–recovery	0.073 ± 0.032	2.26	0.024
	Recovery–baseline	0.467 ± 0.032	14.59	<0.001

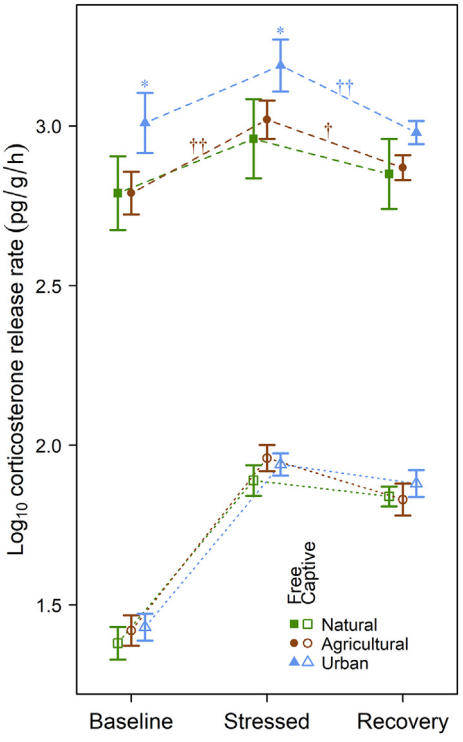

Fig. 1. Corticosterone release rates of tadpoles in the field ("free"; filled symbols) and in the common garden experiment ("captive"; empty symbols) by habitat type of their pond of origin. Error bars represent means and standard errors as predicted by the models in Tables 4–5; the slopes of the lines connecting the error bars illustrate the rates of stress-induced change (baseline to stressed) and negative feedback (stressed to recovery). Asterisks and crosses stand for significant differences in the field between natural ponds and either agricultural or urban ponds for corticosterone release rates (asterisks above the error bars: $^{*}P < 0.05$) or for the rates of stress-induced change and negative feedback (crosses above the lines connecting error bars: $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$).

In the common garden experiment, tadpoles had lower corticosterone release rates but greater stress-induced change compared to free-living tadpoles (Table S5, Fig. 1). The habitat type of their origin had no significant effect on any of the studied hormonal variables (Table 5, Fig. 1). This lack of habitat effect is not attributable to power issues, because power estimation showed that our data had good power for detecting the same differences in the mesocosms as we did in the field (99%, 90%, and 87% power for baseline, stressed, and recovery levels, respectively). In both venues, most of the studied hormone variables varied significantly with date and time of day (Tables 4–5).

4. Discussion

As anthropogenic environmental change continues, there is a growing need to understand how organisms cope with such changes. We explored the glucocorticoid profiles of larval common toads across three habitat types and used a common garden experiment to explore the role of persistent population divergence in these responses. In the free-living tadpoles, we found differences in the rates of both the stress response and its negative feedback associated with land use and water pollution, whereas none of these differences persisted in the common garden.

In accordance with our prediction, our study suggests stronger negative feedback in the HPI axis of common toad tadpoles in anthropogenic habitats than in tadpoles living in natural habitats. Over the 1 h recovery period after the agitation test, tadpoles in both urban and agricultural ponds showed greater downregulation of corticosterone release rates relative to their stressed rates, indicating that their negative feedback had greater scope (magnitude) and/or faster speed

Table 4
Coefficients (b) of GEE models for corticosterone release rates (CORT; $\log_{10}$ pg/g/h), their stress-induced change, and negative feedback in the field.

Dependent variable	Model coefficients[a]	$b \pm$ SE	Wald χ^2	P
Baseline CORT	Natural habitat	2.771 ± 0.098	806.57	<0.001
	Agricultural–natural	0.001 ± 0.089	<0.01	0.996
	Urban–natural	0.217 ± 0.085	6.55	0.011
	Carbamazepine	−0.032 ± 0.036	0.82	0.366
	Corticoid disruptors	0.052 ± 0.083	0.40	0.530
	Date (days from May 1)	−0.015 ± 0.014	1.22	0.269
	Time of day	0.180 ± 0.040	20.16	<0.001
Stressed CORT	Natural habitat	2.906 ± 0.107	744.12	<0.001
	Agricultural–natural	0.067 ± 0.099	0.45	0.502
	Urban–natural	0.230 ± 0.092	6.18	0.013
	Carbamazepine	0.005 ± 0.034	0.02	0.880
	Corticoid disruptors	0.209 ± 0.077	7.38	0.007
	Date (days from May 1)	−0.043 ± 0.013	11.38	0.001
	Time of day	0.221 ± 0.039	31.24	<0.001
Recovery CORT	Natural habitat	2.919 ± 0.097	901.27	<0.001
	Agricultural–natural	0.019 ± 0.083	0.05	0.821
	Urban–natural	0.129 ± 0.085	2.30	0.129
	Carbamazepine	−0.038 ± 0.023	2.77	0.096
	Corticoid disruptors	0.199 ± 0.050	15.82	<0.001
	Date (days from May 1)	−0.053 ± 0.008	46.49	<0.001
	Time of day	0.158 ± 0.026	35.97	<0.001
Stress-induced change[b]	Natural habitat	0.110 ± 0.027	16.26	<0.001
	Agricultural–natural	0.106 ± 0.035	8.98	0.003
	Urban–natural	0.032 ± 0.025	1.61	0.204
	Carbamazepine	0.030 ± 0.012	6.84	0.009
	Corticoid disruptors	0.143 ± 0.015	93.90	<0.001
	Date (days from May 1)	−0.029 ± 0.003	103.18	<0.001
	Time of day	0.051 ± 0.018	7.84	0.005
Negative feedback[c]	Natural habitat	−0.018 ± 0.038	0.24	0.626
	Agricultural–natural	0.066 ± 0.034	3.88	0.049
	Urban–natural	0.103 ± 0.034	9.25	0.002
	Carbamazepine	0.034 ± 0.022	2.45	0.118
	Corticoid disruptors	−0.010 ± 0.053	0.04	0.843
	Date (days from May 1)	0.010 ± 0.008	1.40	0.236
	Time of day	0.080 ± 0.027	8.68	0.003

[a] In each model, the first coefficient is the estimated mean for the natural habitat, whereas the second and third coefficients are the mean differences of agricultural and urban habitats, respectively, from the natural habitat. The remaining coefficients give the estimated change in the dependent variable in response to a unit increase in the predictor variable.
[b] Stress-induced change in CORT was calculated as 100 × (stressed − baseline) / baseline CORT, and transformed to power ¼ after adding the |minimum value − 1|.
[c] Negative feedback was calculated as 100 × (stressed − recovery) / stressed CORT, and transformed to power 4 after adding the |minimum value − 1|.

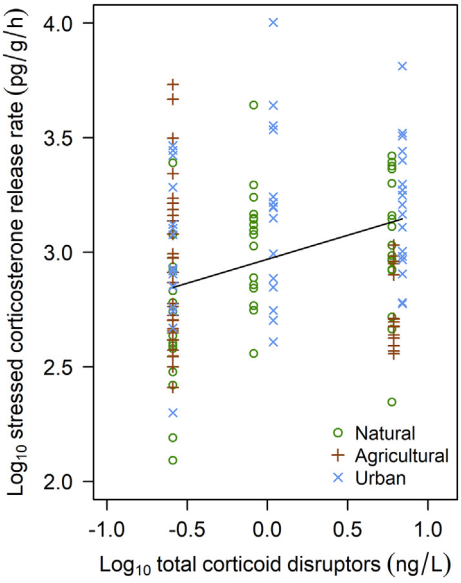

Fig. 2. Stressed corticosterone release rates of free-living tadpoles in relation to the concentration of corticoid disruptors in pond water. The line was fitted from the GEE model in Table 4.

(Taff and Vitousek, 2016) compared to tadpoles in natural habitats. This finding aligns with recent suggestions that the GC negative feedback is an important determinant of the capacity to cope with stress and thus should be favored in populations facing frequent stressors (Narayan et al., 2019; Vitousek et al., 2019; Wingfield, 2013). Although we have no data to assess if the stronger negative feedback is adaptive in anthropogenic habitats, empirical evidence suggests that more efficient negative feedback has fitness benefits under stressful conditions (Romero and Wikelski, 2010; Zimmer et al., 2019). As high GC levels can be detrimental to early-life growth and development in amphibians (Crespi et al., 2013), the ability to quickly shut down the stress response after the stressor ceased should protect the tadpoles from chronic GC elevation and its pathological consequences (Romero et al., 2009; Wingfield, 2013).

This protective effect of strong negative feedback may explain why we, and several previous studies on other species (Bonier, 2012; Sepp et al., 2018), did not find attenuated stress responses in anthropogenic habitats. Although reduced stress responsiveness may also protect the organism from phenotypic damage and the "wear and tear" effect of repeated stress responses (Romero et al., 2009), a weak stress response may be insufficient for adequately dealing with stressful stimuli (Vitousek et al., 2019). A strong stress response may be especially adaptive when the likelihood of unpredictable stressors is high (Romero, 2002), because stress-induced GCs have preparative actions that prepare the organism for a "better" response to a subsequent stressor (Sapolsky et al., 2000), and may increase the threshold of severity necessary for the subsequent stimuli to become stressors (Vera et al., 2017). Thus, instead of dampening the GC stress response, the ability to quickly "turn it on and off" may be the best strategy in stressful environments (Vitousek et al., 2019). Supporting this idea, birds mounting a strong stress response coupled with strong negative feedback are less likely to abandon incubation upon stress (Zimmer et al., 2019) and can breed in disturbed locations (Soldatini et al., 2015). Our tadpole study aligns with these findings, because we found higher stressed corticosterone release rates in urban ponds and proportionally greater stress-induced increase in agricultural than in natural ponds. Currently, it is not clear whether the stressed levels of GC or their stress-induced increase better predict the transcriptomic, phenotypic, and fitness effects of stress (Vitousek et al., 2018). All else being equal, a higher absolute GC concentration has stronger effects (Romero, 2004), suggesting that urban but not agricultural tadpoles responded more to agitation than tadpoles in natural ponds did. However, all else is rarely equal: the effects of GCs depend on other components of the HPA/I axis, including the abundance of GC receptors, corticosteroid binding globulins, and enzymes that metabolize GCs (Breuner et al., 2003; Lattin and Kelly, 2020). For example, long-term elevation of baseline GCs can be accompanied by decreased receptor production and thus diminished biological effects at a given GC concentration (Romero, 2004). Therefore, the increase from baseline to acute stressed GC levels might better express the strength of the stress response when organisms differ in their baseline levels (Vitousek et al., 2018). This scenario, in contrast, suggests that agricultural but not urban tadpoles responded more to agitation than tadpoles in natural ponds did. Because urban but not agricultural tadpoles had higher baseline levels than tadpoles in natural ponds, our results

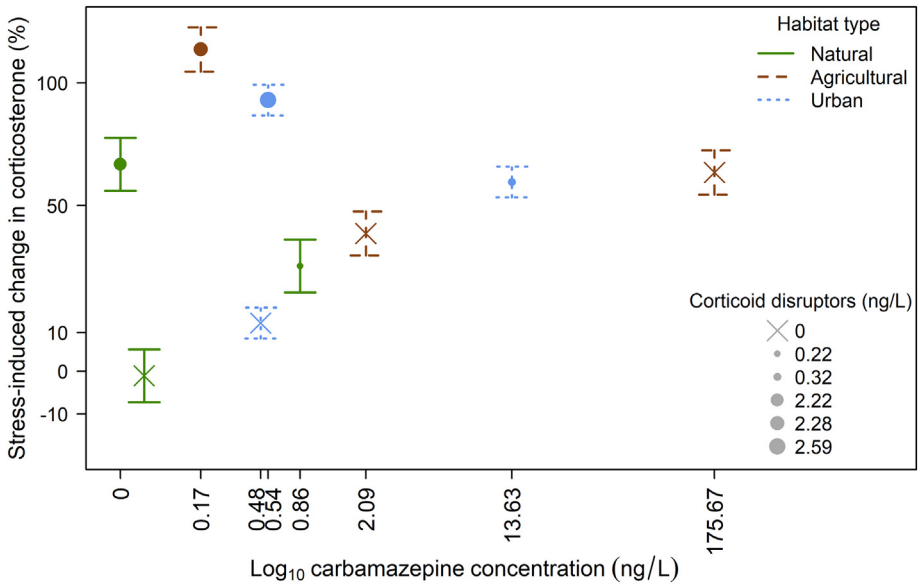

Fig. 3. Stress-induced change of corticosterone release rates in free-living tadpoles in relation to the concentrations of corticoid disruptors and carbamazepine in pond water. Error bars represent means and standard errors as predicted by the model in Table 4. Stress-induced change in corticosterone release rates was calculated as 100 × (stressed − baseline) / baseline. Both axes are shown on transformed scale, as used in the analyses ($Y^{¼}$, $\log_{10}X$).

on stressed levels and stress-induced change may altogether suggest stronger stress response in both types of anthropogenic habitats. We propose that stronger negative feedback allows for stronger stress responses in such habitats, offering an alternative (and potentially more advantageous) strategy for minimizing the time spent at high GC levels; and we suggest that this may be a widespread reason for not finding a dampened stress response in urban animals.

We also observed higher baseline corticosterone release rates of tadpoles in urban ponds than in the tadpoles in the other habitat types. Elevated baseline GC levels are often interpreted as a sign of chronic stress; however, chronic stress can either increase or decrease or have no effect on baseline GC levels (Dickens and Romero, 2013). Pathogenic infections may also elevate GC levels (Gabor et al., 2015, 2013; Raouf et al., 2006; Warne et al., 2011), but we detected neither *Ranavirus* nor *Bd* in our tadpoles even though both are present in Hungary (Vörös et al., 2020, 2018). Alternatively, higher baseline levels may be adaptive in several, mutually non-exclusive ways. Baseline GCs have permissive effects that allow the organism to perform better under stress (Sapolsky et al., 2000), thus temporal variation in baseline GC levels has been proposed to serve as preparation for periods of high potential exposure to adverse conditions (Romero, 2002). Similarly, spatial variation in the likelihood of exposure to stressful stimuli may explain the difference we observed between tadpole populations. Furthermore, because GC levels are upregulated during times of increased energetic demands (Romero, 2002), it is possible that urban tadpoles need a higher baseline for maintaining a higher metabolic rate. For example, urban ponds are often more polluted (Bókony et al., 2018), which may favor higher detoxification rates, thereby increasing energy demands. In our present study, concentrations of a general wastewater marker, carbamazepine were relatively high in urban ponds, although they were also high in two agricultural ponds where baseline corticosterone release rates were not elevated, and we found no correlation between baseline corticosterone release rates of tadpoles and carbamazepine concentrations across our ponds. However, urban ponds had higher conductivity, total dissolved solids, and salinity, suggesting contamination by other toxicants such as de-icing salts, which can increase baseline corticosterone levels in amphibians (Chambers, 2011; Goff et al., 2020; Hall et al., 2017). Furthermore, higher metabolic rates may also result from the urban heat island effect which makes urban ponds warmer than rural ponds (Brans et al., 2018).

We found no close relationship between anthropogenic land use and carbamazepine concentrations, and the total amount of corticoid-disrupting compounds varied independently of both land use and carbamazepine (reflecting overall pollution) levels. In turn, each of these three aspects of anthropogenic environmental change had some effects on the tadpoles' corticosterone profiles. The concentrations of both carbamazepine and corticoid disruptors were positively correlated with the stress-induced change, and more corticoid disruptors in pond water were accompanied by higher stressed corticosterone release rates in tadpoles. These results suggest that various chemical contaminants may contribute to altered GC physiology even in habitats where land use does not indicate strong anthropogenic influence, supporting previous findings that even non-anthropogenic ponds can be contaminated by various endocrine disruptors (Bókony et al., 2018). Furthermore, we did not find increased prevalence of two major amphibian pathogens in anthropogenic ponds, despite that both pathogens are presumed to be vectored in association with human activities (St-Amour et al., 2008) and are found throughout Hungary (Vörös et al., 2020, 2018). These results highlight that the stressors faced by wild animals may vary in complex ways across gradients of anthropogenic environmental change.

Our common garden experiment showed that the differences observed in free-living tadpoles' corticosterone profiles did not persist when the animals were raised in captivity in uncontaminated water, suggesting that individual phenotypic plasticity was responsible for the differences in the field, rather than persistent divergence between populations. This finding contrasts the results of two previous studies on birds, which suggested that evolutionary divergence or other transgenerational (e.g., maternal or epigenetic) effects were responsible for generating differences in GC stress response between urban and non-urban populations (Atwell et al., 2012; Partecke et al., 2006), although a third avian study found that phenotypic plasticity played a more important role in generating such differences in baseline GC levels (Ouyang et al., 2019). Our present results parallel the findings of an earlier study (conducted with partly the same tadpole populations), which showed higher chemical defense in toads at urban and agricultural sites that did not persist in a common garden experiment (Bókony et al., 2019). A potential explanation for this apparent importance of phenotypic plasticity is that anthropogenic environments may exert complex selection forces on tadpole physiology because of spatio-temporal

Table 5
Coefficients (b) of GEE models for corticosterone release rates (CORT; $\log_{10}$ pg/g/h), their stress-induced change, and negative feedback in the common garden experiment.

Dependent variable	Model coefficients[a]	$b \pm$ SE	Wald χ^2	P
Baseline CORT	Natural habitat, May 6	0.952 ± 0.041	530.12	<0.001
	Agricultural–natural	0.044 ± 0.069	0.40	0.530
	Urban–natural	0.054 ± 0.066	0.67	0.410
	Date (May 7–May 6)	1.086 ± 0.115	88.71	<0.001
	Date (May 8–May 6)	1.061 ± 0.140	57.70	<0.001
	Time of day	0.569 ± 0.128	19.78	<0.001
Stressed CORT	Natural habitat, May 6	1.651 ± 0.059	777.69	<0.001
	Agricultural–natural	0.076 ± 0.062	1.49	0.223
	Urban–natural	0.053 ± 0.058	0.83	0.361
	Date (May 7–May 6)	0.593 ± 0.106	31.57	<0.001
	Date (May 8–May 6)	0.529 ± 0.105	25.22	<0.001
	Time of day	0.275 ± 0.109	6.35	0.012
Recovery CORT	Natural habitat, May 6	1.482 ± 0.041	1317.94	<0.001
	Agricultural–natural	−0.013 ± 0.058	0.05	0.817
	Urban–natural	0.036 ± 0.052	0.48	0.486
	Date (May 7–May 6)	0.601 ± 0.127	22.33	<0.001
	Date (May 8–May 6)	0.831 ± 0.131	40.05	<0.001
	Time of day	0.223 ± 0.107	4.40	0.036
Stress-induced change[b]	Natural habitat, May 6	0.698 ± 0.073	91.60	<0.001
	Agricultural–natural	0.046 ± 0.093	0.24	0.624
	Urban–natural	0.001 ± 0.084	<0.01	0.993
	Date (May 7–May 6)	−0.522 ± 0.146	12.79	<0.001
	Date (May 8–May 6)	−0.545 ± 0.185	8.71	0.003
	Time of day	−0.322 ± 0.156	4.28	0.039
Negative feedback[c]	Natural habitat, May 6	0.182 ± 0.055	11.10	0.001
	Agricultural–natural	0.059 ± 0.071	0.69	0.407
	Urban–natural	0.028 ± 0.060	0.23	0.634
	Date (May 7–May 6)	0.044 ± 0.113	0.15	0.695
	Date (May 8–May 6)	−0.262 ± 0.115	5.16	0.023
	Time of day	0.115 ± 0.119	0.93	0.336

[a] In each model, the first coefficient is the estimated mean for tadpoles from the natural habitat on May 6, whereas the second and third coefficients are the mean differences of tadpoles from agricultural and urban habitats, respectively, from the natural habitat. Similarly, coefficients for date are the differences between May 6 and later days. Coefficients for time of day give the estimated change in the dependent variable in response to an hour increase in time.

[b] Stress-induced change in CORT was calculated as 100 × (stressed − baseline) / baseline CORT, and transformed to power ¼ after adding the |minimum value − 1|.

[c] Negative feedback was calculated as 100 × (stressed − recovery) / stressed CORT, and transformed to power 4 after adding the |minimum value − 1|.

heterogeneity in the frequency and type of stressors, which should then favor the evolution and maintenance of phenotypic plasticity (Bradshaw and Hardwick, 1989; Moran, 1992; Sultan and Spencer, 2002). Alternatively, evolutionary adaptation may be constrained by low heritability, although the latter seems unlikely for GCs which are heritable in various species (Guindre-Parker, 2018).

An alternative explanation for our results may be genotype-by-environment interaction (G × E). Specifically, it is possible that the GC differences between free-living populations were due to genetic differences that were expressed in the wild but did not get expressed in the captive environment. Although we cannot rule out this possibility, G × E is a less parsimonious explanation for our findings than phenotypic plasticity alone, because G × E requires not one, but two, processes: genetic differentiation between populations (explaining our field results) and phenotypic plasticity (explaining the lack of phenotypic differences in the common garden). While there is empirical evidence that genetic differences may be "hidden" by differences in phenotypic plasticity, increased phenotypic variability due to latent genetic variability often becomes expressed when the organisms are taken out from the environment to which they have adapted (DeWitt and Scheiner, 2004). This pattern is the opposite of what we found, since the GC differences between populations diminished, rather than increased, in the common garden. Nevertheless, further study is needed to explicitly test the role of G × E, by performing the common-garden experiment in several different environments.

Taken together, our study demonstrated marked differences between natural and anthropogenic habitats in the GC stress physiology of toad tadpoles, which were related partly to land use and partly to chemical pollution. Both urban and agricultural populations showed stronger negative feedback, and by some measures also stronger stress response, compared to populations in natural habitats, supporting the idea that dynamic regulation of the GC stress response is an important component of stress coping capacity that is favored in anthropogenic environments (Narayan et al., 2019; Vitousek et al., 2019). Our common garden experiment demonstrates that the differences observed between populations are unlikely to have resulted from microevolution or transgenerational effects. Altogether, our results suggest that toad tadpoles upregulate their GC negative feedback efficiency, a major component of endocrine flexibility, by phenotypic plasticity in response to stressors in anthropogenic environments. To understand the consequences of these changes, more research will be needed to elucidate how the scope and speed of endocrine flexibility at multiple levels of GC regulation (Guindre-Parker, 2018; Lattin and Kelly, 2020; Taff and Vitousek, 2016; Vitousek et al., 2019) affect individual fitness and population viability in our human-modified world.

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CRediT authorship contribution statement

Veronika Bókony: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - original draft, Supervision, Funding acquisition. **Nikolett Ujhegyi:** Investigation, Writing - review & editing. **Kamirán Á. Hamow:** Investigation, Writing - review & editing. **Jaime Bosch:** Investigation, Writing - review & editing. **Barbora Thumsová:** Investigation, Writing - review & editing. **Judit Vörös:** Investigation, Writing - review & editing. **Andrea S. Aspbury:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Caitlin R. Gabor:** Conceptualization, Methodology, Investigation, Supervision, Visualization, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.141896.

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